

*Review*

# Cryopreservation of plant genetic resources: A legacy for humanity

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**Plant genetic resources are essential for agri-food security in the world and conservation of genetic diversity. Cryopreservation is an *in vitro* culture technique used for long-term plant conservation, by freezing the tissue at low temperatures usually with liquid nitrogen (-196°C). During cryopreservation, cell division and metabolic activity of the explants are quiescent. There are different cryopreservation techniques used for many species and recently it has been observed that the use of aluminum cryoplates or foils increase plant survival and regeneration. The explants are exposed to a lot of stress in the different stages of cryopreservation, especially during chemical or physical dehydration and during thawing. Cryopreserved plants are exposed to physical, chemical and physiological cell damage and oxidative stress. The principal cause of plant cell mortality is membrane rupture due to ice crystal formation. The cryoprotective substances prevent ice formation and optimal dehydration is necessary for plant survival and regeneration. Different cryopreservation stages could alter genetic stability, especially during plant regeneration by the use of plant growth regulators. DNA alteration during *in vitro* culture depends on different factors, mainly cryopreservation technique and plant species. Molecular markers are used to detect variations in the DNA of cryopreserved plants. A successful cryopreservation protocol depends on survival, regeneration and genetic stability of plant materials.**

**Key words:** Cryopreservation, plant genetic resources, cryogenic damage, plant regeneration, genetic stability.

## INTRODUCTION

Plant genetic resources (PGR) are any vegetal genetic materials with real or potential value for agri-food security (Sonnino, 2017). Only 30 crops cover 95% of global agri-food needs and these have been used to produce new varieties, which have important characteristics such as higher production and tolerance to biotic and abiotic

stresses (Kaviani, 2011; Shiferaw et al., 2011; FAO, 2021). Therefore, it is essential to preserve PGR because they represent a great part of plant biodiversity.

The preservation of plant species depends greatly on whether their seeds are of orthodox or recalcitrant nature and the latter species are often conserved in field

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collections (Guzmán et al., 2017). Nevertheless, PGR maintained in field are affected by biotic and abiotic factors (Niino and Arizaga, 2015). Thus, an alternative is *in vitro* medium or long-term conservation. *In vitro* plant culture requires multiple techniques that enable the growth of cells, tissues and organs in aseptic culture media (Oseni et al., 2018).

Cryopreservation consists of the interruption of metabolic functions of biological materials by decreasing the temperature with liquid nitrogen (LN) (-196°C), while maintaining viability (Niino and Arizaga, 2015). Cryopreservation in liquid nitrogen is an alternative for long-term conservation of PGR (Panis, 2019). During cryopreservation, the cell cycle and metabolic and biochemical activity are detained, therefore, the biological material can be safeguarded for practically indefinite periods (Benson et al., 2006). After cryopreservation, plant materials will recover and regenerate into a plant, depending on treatments before and after exposure to LN and there is a minimal risk of DNA alterations (Adu-Gyamfi et al., 2016; Nuc et al., 2016; Gross et al., 2017). The genetic stability of cryopreserved plant material is assessed with morphological, cytological, biochemical and, for the most part, molecular markers (Harding, 2004).

Different cryopreservation protocols have been developed for various species using different types of explants such as seeds (Schofield et al., 2018), synthetic seeds (Petrus et al., 2019), shoots (Bruňáková and Čellárová, 2016), apices (Liu et al., 2017), pollen (Souza et al., 2018), embryogenic cultures (Varis et al. 2017), zygotic embryos (le Roux et al. 2016) and cell suspensions (Titova et al., 2021). The choice of explant used for cryopreservation is dependent on the objective; and while seeds and embryos are the main source of genetic diversity, shoot tips and dormant buds are clonal materials (Reed, 2017).

The response of plant material depends on the species and its genetic variability, explant type, cryopreservation technique as well as the stressful environment of tissue culture (Kaya and Souza, 2017; Popova and Kim, 2019; Bednarek and Orłowska, 2020). Therefore, plant cryopreservation protocols developed, are specific for each plant material and focus on achieving the maximum post-cryogenic survival and regeneration (Harding, 2004).

## TECHNIQUES USED IN CRYOPRESERVATION

Cryopreservation techniques are classified into conventional and new techniques. The first ones are based on the partial chemical dehydration of the explants with osmoprotectors followed by gradual freezing with automatic freezers (Engelmann, 2011). The new techniques are based on vitrification and the use of aluminum cryoplates that facilitate the manipulation of the explants as well as faster cooling and heating

(Matsumoto, 2017).

Plant vitrification requires a highly concentrated solution that sufficiently dehydrates tissues and solidifies intracellular water into metastable glass to avoid crystallization in the cell (Matsumoto, 2017). On the other hand, the aluminum cryoplates have been successful to obtain high shoot regeneration, greater than 90% in various species (Yamamoto et al., 2015; Rafique et al., 2016; Dhungana et al., 2017; Thammasiri et al., 2019; Pettinelli et al., 2020; Zhang et al., 2020; Benelli et al., 2021; Tanaka et al., 2021). Table 1 shows the main plant cryopreservation methods developed in different species. There are many combinations of procedures carried out in cryopreservation techniques like encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pre-growth, pre-growth-drying and droplet-vitrification (Engelmann, 2004).

## Stages of cryopreservation

Cryopreservation includes different stages depending on the selected technique, involving preparation and explant excision, preculture, cryoprotection, vitrification/dehydration, fast cooling in LN, rewarming, cryoprotector elimination, regeneration and plant culture (Volk et al., 2004; Liu et al., 2017; Streczynski et al., 2019). These stages are schematized in Figure 1. The critical point is an optimum dehydration to avoid lethal ice crystals formation in plant cells (Pence et al., 2020). To reduce explant water content, air-drying in laminar flow hood, silica gel, cryoprotectants, slow cooling and preculture are used (O'Brien et al., 2021). The selection depends on explant tolerance to stress, and these can be used singly or combined.

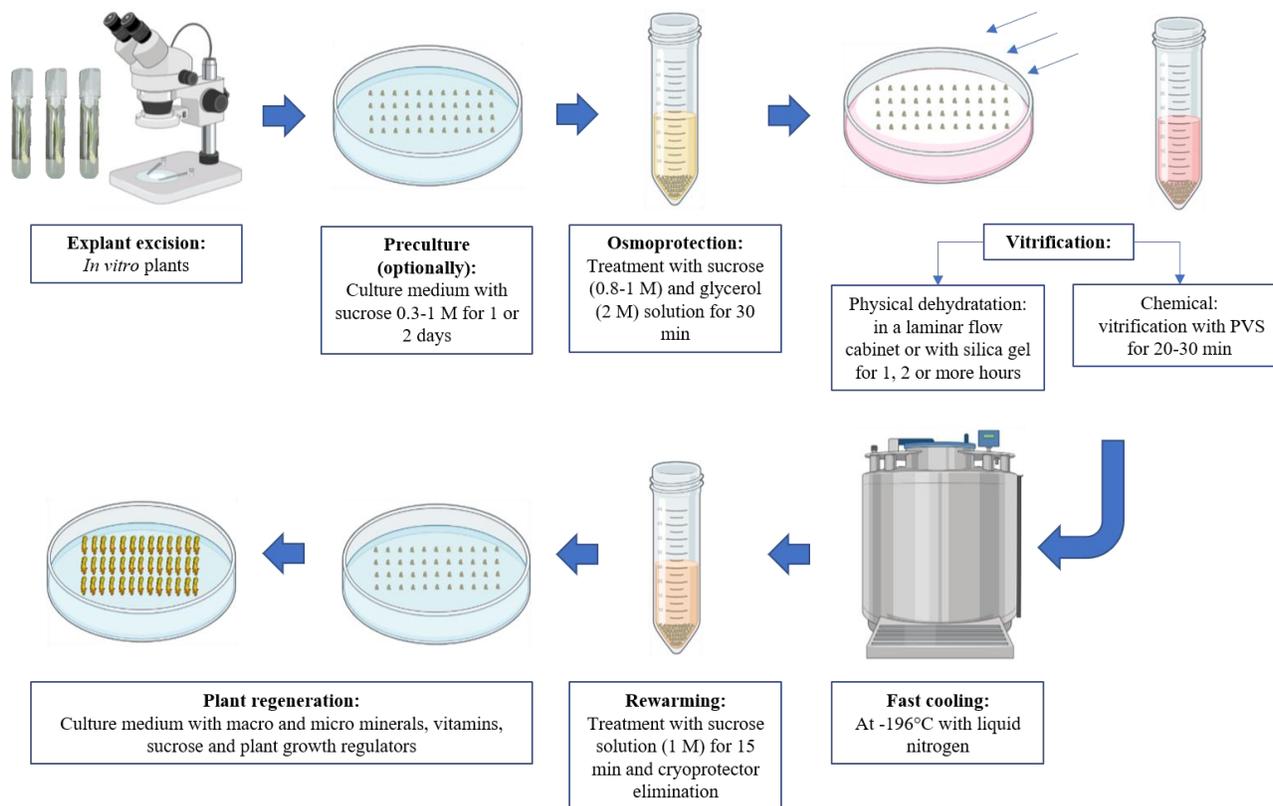
## MAIN COMPLICATIONS DURING CRYOPRESERVATION

Cryopreservation causes stress in plant cells that are subjected to excision, osmotic dehydration and sudden changes in temperature (Uchendu et al., 2013). This stress reduces plants survival and regeneration (Lynch et al., 2011). Freezing injuries affect cell membranes that lose the fluidity of their lipid components (Centinari et al., 2016). Biological membranes define cell limits and internal organelles; they are highly dynamic which allows the maintenance of integrity, resistance to stress, flexibility, fluidity and electrical insulation (Gould, 2018). Plant membranes are constituted mainly of galactolipids, phospholipids and lysophospholipids. Some classes with large polar head groups are phosphatidylcholine and digalactosil diacylglycerol, while others have small head groups such as phosphatidylethanolamine, monogalactosyldiacylglycerol, and phosphatidic acid (Lin et al., 2021).

**Table 1.** Main plant cryopreservation methods developed in different species.

Cryopreservation methods	Species	Preculture	Osmoprotection	Vitrification	Rewarming	Plant regeneration	Reference
Vitrification	Apical meristems of <i>Chlorophytum borivillianum</i>	MS medium with 0.5 M glycerol for 48 h at 25°C	18.4% glycerol and 13.7% sucrose for 20 min	PVS2 for 30 min at 0°C	40% sucrose for 10 min	33%	Chauhan et al. (2021)
Pregrowth-dehydration	Embryogenic tissue of <i>Picea abies</i> L.	0.25-1 M sucrose and 10 µM ABA for 7 days	N/A	Dehydration with silica gel for 2 h at 25°C	Water bath at 42°C for 3 min, after on ice for 2-3 min and 1-0 M sucrose for 1.5 h	276 embryos g <sup>-1</sup>	Hazubska-Przybył et al. (2013)
D-Crio-plate (vitrification by air current on aluminium cryo-plates)	Buds of <i>Juncus decipiens</i> Nakai	MS medium with 0.3 M sucrose overnight at 25°C	2 M glycerol and 1.0 M sucrose solution for 30 min	Air current of a laminar flow cabinet for 3 h at 25°C	1 M sucrose for 15 min	90%	Niino et al. (2013)
Air drying (flash drying, normal drying)	Zygotic embryos of <i>Seemannaralia gerrardii</i>	Electrolysed water of CaMg solution (0.5 mM CaCl <sub>2</sub> .2H <sub>2</sub> O and 0.5 mM MgCl <sub>2</sub> .6H <sub>2</sub> O) for 30 min in the dark	N/A	Flash-dried for 50 min at 25°C	Electrolysed water of CaMg solution (0.5 mM CaCl <sub>2</sub> .2H <sub>2</sub> O and 0.5 mM MgCl <sub>2</sub> .6H <sub>2</sub> O) for 30 min in the dark	70%	Berjak et al. (2011)
V-Crio-plate (vitrification and aluminium cryo-plates)	Shoot tips of <i>Tanacetum cinerariifolium</i>	MS medium with 0.5 M sucrose for 2 days at 5°C	2 M glycerol and 1.4 M sucrose solution for 30-60 min	PVS 7M for 40 min at 25°C	1 M sucrose for 15 min	90%	Yamamoto et al. (2011)
Droplet vitrification (droplets of cryoprotectant on an aluminium foil strip)	Meristems of <i>Musa</i> spp.	MS medium with 3, 6 y 9% sucrose for 1-2 days at 25°C	2 M glycerol and 0.4 M sucrose solution for 20 min	PVS2 for 30 min at 0°C	1.2 M sucrose for 15 min	52.9%	Panis et al. (2005)
Pregrowth	Meristems of <i>Musa</i> spp.	MS medium with 0.4 M sucrose and 1 µM IAA for 2 weeks at	N/A	N/A	Water bath at 40°C for 1.5 min.	66%	Panis et al. (2002)
Encapsulation-dehydration	Apices of <i>Wasabia japonica</i>	½ MS medium with 0.3 M sucrose for 1 day at 20°C	Encapsulation into 2% Na-alginate beads containing 0.4 M sucrose and treated with MS medium and 0.8 M sucrose solution for 16 h at 25°C	Dehydration with silica for 4 h at 25°C	Water bath at 35°C for 3 min	75%	Sakai et al.,(2000)
Encapsulation-vitrification	Apices of <i>Mentha spicata</i>	MS medium with 30 g L <sup>-1</sup> sucrose for 3 weeks at 4°C	Encapsulation into 2% Na-alginate beads containing 0.4 M sucrose and treated with 2 M glycerol and 0.4 M sucrose solution for 1 h	PVS2 for 3 h at 0°C	Water bath at 38°C for 1 min and MS medium with 1.2 sucrose for 10 min	87%	Sakai et al. (2000)

ABA: Abscisic acid. IAA: Indole-3-acetic acid. MS: Murashige and Skoog (1962). N/A: Does not apply. PVS: Plant Vitrification Solution (30% glycerol, 19.5% ethylene glycol and 0.6 M sucrose). PVS2: 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide and 0.4 M sucrose.



**Figure 1.** Stages of cryopreservation. PVS: Plant vitrification solution.

### Cryogenic damage

Temperature variations affect the lipid composition of membranes and therefore their biophysical properties (Cook et al., 2021). The integrity of the plasma membrane is a factor on which cell viability depends because it is considered a primary site of cryogenic injury. The mortality of plant explants is mainly due to improper thawing which causes cryogenic damage on cell membrane (Yang et al., 2017). In cryopreserved maize zygotic embryos, different degrees of cell lesions have been observed. The main symptoms include plasmolysis, mitochondrial condensation, increased heterochromatin, nuclear contraction and chromatin condensation, as well as rupture of the cell wall, cell membrane and nuclear envelope (Wen et al., 2010).

### Oxidative stress

Cryopreservation procedures can cause physical, chemical and physiological cell damage in addition to oxidative stress (Martín and González-Benito, 2005). The first immediate response to biotic and abiotic stress in plant cells is formation of reactive oxygen species (ROS) and these tend to decline when the stress disappears

(Huang et al., 2019). ROS are free radicals like superoxide anion ( $O_2^{2-}$ ), hydroperoxyl radical ( $OH_2^{\cdot}$ ), alkoxy radical ( $RO^{\cdot}$ ) and hydroxyl radical ( $^{\cdot}OH$ ), also nonradical molecules such as hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ) (Mehla et al., 2017; Hasanuzzaman et al., 2019). These signaling molecules also regulate plant metabolism and are produced in organelles like chloroplast, mitochondria, peroxisome and cytoplasm (Das et al., 2015; Huang et al., 2019). However, an excessive concentration of intracellular ROS leads to oxidative stress and damage to lipids, proteins and DNA; therefore, it alters the plasma membrane and metabolic pathways, ending in cell death (Nita and Grzybowski, 2016).

Favorably, plant cells have enzymatic and non-enzymatic antioxidant systems that maintain ROS homeostasis (You and Chan, 2015). The enzymatic antioxidant systems are superoxide dismutase, catalase and peroxides involved in the reduction process of  $O_2^{\cdot-}$  to  $H_2O_2$ . The non-enzymatic components are generally ascorbic acid,  $\alpha$ -tocopherol, flavonoids, glutathione, carotenoids, lipids, and phenolic compounds, which are efficient antioxidants (Nadarajah, 2020).

However, even when antioxidant substances are used during cryopreservation, these systems are often impaired during freezing and thawing (Kaczmarczyk et

al., 2012). The factors associated with oxidative stress during cryopreservation are diverse, including disinfection agents, explant cutting, osmotic agents, freezing and thawing (Chen et al., 2014). Particularly, plant tissues are exposed to cell lysis during aseptic establishment process and this causes the synthesis and release of phenolic compounds, whose high accumulation is toxic and causes cell death (Jones and Saxena, 2013).

The most common disinfection agents used are sodium hypochlorite (Bello et al., 2018), mercuric chloride (Haider et al., 2015), ethanol (Maina et al., 2010), ozone (Cabrera Jova and González, 2014), antibiotics and commercial fungicides (Ray and Ali, 2016). Surface disinfectants cause oxidative stress and cell death depending on the concentration used, contact time and type of plant explant (Cuba-Díaz et al., 2020).

During cryopreservation, direct mechanical damage causes cell wall injury and produces an explosion of ROS composed primarily of hydrogen peroxide (Whitaker et al., 2010; Skyba et al., 2012). Excess of hydrogen peroxide triggers autophagy by chloroplasts and peroxisomes as well as programmed cell death (Smirnov and Arnaud, 2019).

On the other hand, oxidative stress caused by cryoprotectants induces lipidic peroxidation and oxidative phosphorylation damage (Ren et al., 2013). Lipid peroxidation is the decomposition of lipids into aldehydes such as 4-hydroxynonenal and malondialdehyde under the action of ROS (Liu et al., 2021). Cryoprotectants like polyethylene glycol, mannitol and sucrose are osmotic stress agents under *in vitro* culture conditions (Şen, 2012). In addition, sorbitol pretreatments increase intracellular hydrogen peroxide concentrations (Lynch et al., 2011).

Plant cells are exposed to osmotic stress during the osmoprotection and dehydration process, which can lead to excessive ROS release (Whitaker et al., 2010; Rahmah et al., 2015). Osmotic dehydration involves treating tissues with concentrated sugar solutions or other osmotically active substances, which allows water to flow through membranes from a lower solute concentration to a higher concentration (Volk and Walters, 2006).

Therefore, plants need to increase the cryostability capacity of the plasma membrane to tolerate various stresses caused by freezing and thus accelerate the recovery process after thawing (Uemura et al., 2009).

## CRYOPROTECTIVE AGENTS

Cryoprotective agents (CPAs) are high or low molecular weight water-soluble substances that facilitate dehydration and decrease the osmotic gradient of cells through vitrification during cryopreservation and long-term storage in liquid nitrogen (Yang et al., 2017).

The function of CPAs is to prevent ice formation and

cryogenic damage through interference of hydrogen bonds in water molecules (Towey and Dougan, 2012). Most of the time, plant survival depends on optimal dehydration with CPAs (Elliot et al., 2017).

Permeable CPAs to plasma membrane are low molecular weight and the most widely used are methanol, dimethyl sulfoxide, glycerol, propylene glycol, ethylene glycol and formamide. Non-permeable CPAs are of high molecular weight, among which are polyvinylpyrrolidone, hydroxyethyl starch and some sugars (Gurruchaga et al., 2018).

However, the toxicity of penetrating CPAs is an obstacle to successful cryopreservation (Streczynski et al., 2019). The CPAs toxicity is associated with temperature, CPAs concentration, time of exposure, plant species and tissue development (Rahmah et al., 2015). CPAs are toxic when they break membranes, alter enzyme function or if cell viability is decreased (Best, 2015).

## PLANT REGENERATION

Plant regeneration after a cryopreservation process will depend mainly on the species, explant type and the cryopreservation technique used (Mathew et al., 2018). The time required for plant regeneration is variable; in some cases, it takes a few months and, in others, it takes up to a year or more to obtain fully acclimatized plants (Kaczmarczyk et al., 2012).

Regeneration rates are also influenced by the sensitivity of plant tissue to various types of cryopreservation stress (Uchendu et al., 2013). Therefore, before and after freezing, specific solutions are used to cryoprotect cells and prevent or reduce recrystallization (Yang et al., 2017) which reduces negative impact of cryopreservation stress in cells.

Another important aspect during plant regeneration after cryopreservation is the genetic stability, because the aim of plant genetic resources conservation is to ensure the true-to-type status of the regenerants (Wang et al., 2021).

### Genetic stability of cryopreserved plants

During plant cryopreservation, genetic stability evaluation helps to verify if the DNA from cryopreserved plants remains intact (Dar et al., 2019). In general, nulls or minimal differences have been observed in plant materials before and after cryopreservation (Matsumoto et al., 2013).

However, freezing and thawing could alter lipids and proteins, which can cause chromosomal damage and induce genetic and epigenetic changes (Chatterjee et al., 2016). Other factors that could alter DNA are plant growth regulators used for plant regeneration after

**Table 2.** Evaluation of genetic stability in plant cryopreservation.

Molecular marker	Species	Cryopreservation technique	Genetic stability	Reference
MSAP, RAPD and AFLP markers	<i>Mentha x piperita</i> L.	Encapsulation-dehydration	MSAP results revealed variation in the DNA methylation pattern of mint apices after cryopreservation. RAPD and AFLP markers showed a complete genetic stability.	Ibáñez et al., 2019)
Transcript expression patterns and qPCR methods	<i>Arabidopsis thaliana</i> (cv. Columbia)	Vitrification (PVS2 and PVS3)	Changes in transcript expression in shoot tips.	Gross et al. (2017)
Microsatellites	<i>Picea abies</i> L. Karst	Slow-cooling	Identical prior to and following cryopreservation of somatic embryos.	Varis et al. (2017)
ISSR markers	<i>Malus domestica</i>	Two-step freezing	No polymorphism found between the mother plant and regenerants before and after cryopreservation of apple dormant buds.	Yi et al. (2015)
RAPD markers	<i>Eutrema japonicum</i> Matsum.	Vitrification (PVS2)	No significant differences were observed using RAPD PCR in wasabi plants regenerated from shoot tips after cryopreservation.	Matsumoto et al. (2013)
RAPD markers	<i>Prunus armeniaca</i> L.	Encapsulation-dehydration	No changes in genetic stability in apricot shoot tips after cryopreservation.	Soliman (2013)

AFLP: Amplified Fragment Length Polymorphism. ISSR: Inter Simple Sequence Repeat. MSAP: Methylation Sensitive Amplification Polymorphism. qPCR: Real-Time Polymerase Chain Reaction. PVS: Plant Vitrification Solution. RAPD: Random Amplified Polymorphic DNA.

cryopreservation; these could induce somaclonal variation in the regenerated plants (Bairu et al., 2011; Sales and Butardo, 2014; Butiuc-Keul et al., 2016).

Furthermore, different stages of cryopreservation affect cell functionality, protein expression and DNA stability in different ways (Chatterjee et al., 2016). In general, substances used in pretreatment, preculture and cryoprotection stages could affect genetic stability of explants (Martín et al., 2011). Specifically, histone methylation and modification have been observed during preculture and vitrification (Heringer et al., 2013). Likewise, increased methylation has been reported in tissue culture associated with the use of CPAs and stress conditions (Smulders and de Klerk, 2011; Orłowska et al., 2016; Gross et al., 2017; Ibáñez et al., 2019).

Molecular markers are used to detect variations or polymorphisms between individuals for specific regions of DNA. These polymorphisms can be used to construct genetic maps and evaluate differences between markers in the expression of

traits in a population (Marwal et al., 2014).

Some molecular markers that have been widely used in plant cryopreservation are presented in Table 2. Nevertheless, DNA alteration during *in vitro* culture depends on the species, genotypes and culture conditions (Surenciski et al., 2007).

## CONCLUSION

The bases of food security are PGR because they provide a source of energy, fiber and metabolites of industrial and pharmaceutical interest. Cryopreservation is a viable alternative to safeguard these valuable resources. Plant cryopreservation is a multiple stage process and there are different plant cryopreservation techniques that have been used for different explants. Nevertheless, different responses have been observed because there are many factors that influence the development of plants after cryopreservation protocols in such a way that the existing protocols are specific for each species and genotypes. ROS production is the primary

explant response after cryopreservation, and it has been associated with low plant survival and regeneration. In this sense, avoiding ice crystal formation through CPAs is crucial during osmoprotection.

The success of a cryopreservation protocol depends on survival, regeneration and genetic stability of the plant material. These are fundamental variables for the development of suitable cryopreservation protocols for plant germplasm.

It is necessary to develop cryopreservation protocols for species of agri-food interest that are not yet cryopreserved, wild relatives and for those that are at risk of loss. In addition, it is necessary to optimize existing protocols for their application in different genotypes.

The use of cryopreservation for long-term PGR conservation contributes to ensure agricultural and food security for many generations to come, either through the use of these resources to repopulate or to carry out genetic improvement and generate varieties better adapted to the challenges ahead.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ABBREVIATIONS

PGR, Plant genetic resources; NL, liquid nitrogen; ROS, reactive oxygen species; **CPAs**, cryoprotective agents.

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